Cripto-1 Plasmid DNA Vaccination Targets Metastasis and Cancer Stem Cells in Murine Mammary Carcinoma

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Conflict of interest:

R.Kiessling is in the Scientific Advisory Board for the companies Immunicum AB, Idogen Inc, Glactone Pharma AB and RXi Pharma, and a board member of Clinical Laserthermia Systems AB, and has received compensation for arranging courses for Bristol Myers Squibb and a research grant from Moderna Therapeutics.

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Statement of translational relevance

Despite the wide range of therapies approved for treatment of breast cancer, mortality of patients due to metastatic spread has not been yet been addressed. The development of metastasis targeting treatments is essential in decreasing breast cancer related deaths in long term. Here we describe a vaccine based therapeutic approach targeting tumor antigen Cripto-1 expressed on tumor cells. We show that vaccination with Cripto-1 encoding DNA elicits an anti-Cripto-1 directed immune response that consequently controls metastasis. Cripto-1 expression has also been found on cancer stem cell like cells. Cancer stem cells are highly resistant to chemo and radiotherapy. They can be the cause for relapse and metastases due to their persistence after standard treatment. The anti-Cripto-1 directed immune response was able to eliminate cancer stem cells. Taken together, our data shows great potential of targeting tumor associated antigen Cripto-1 in controlling metastasis and eliminating cancer stem cells.

Abstract

Purpose: Metastatic breast cancer is a fatal disease responding poorly to classical treatments. Cancer vaccines targeting antigens expressed by metastatic breast cancer and cancer stem cells have the potential to become potent anti-cancer therapies. Cripto-1 is an onco-fetal protein frequently overexpressed in invasive breast cancer and cancer-initiating cells. In this study, we explored the potential of a Cripto-1 encoding DNA vaccination to target breast cancer in preclinical models.

Experimental Design: BALB/c mice and BALB-neuT mice were treated with a DNA vaccine encoding for mouse Cripto-1 (mCr-1). Mice were challenged with murine breast cancer 4T1 cells or TUBO spheres, or spontaneously developed breast cancer in the BALB-neuT model. Tumor growth was followed in all mouse models and lung metastases were evaluated. In-vitro assays were performed to identify the immune response elicited by vaccination.

Results: Vaccination against mCr-1 reduced primary tumor growth in the 4T1 metastatic breast cancer model and significantly reduced lung metastatic burden. The primary tumors in the BALB-neuT model are Cripto-1 negative. Consequently, we did not observe protection regarding the primary tumors. However, vaccination significantly reduced lung metastatic burden in this model. Spheroid cultured TUBO cells, derived from a BALB/neuT primary tumor, obtain cancer stem cell like phenotype and upregulate m-Cr-1. We observed reduced tumor growth in vaccinated mice after challenge with TUBO spheres.

Discussion: Our data indicates that vaccination against Cripto-1 results in a protective immune response against mCr-1 expressing and metastasizing

tumors. Targeting Cripto-1 by vaccination is a promising potential immunotherapy for treatment of metastatic breast cancer.

Introduction

Breast cancer is the most common cancer among women in western countries and incidence rates have been rising in developing countries in the last years (1). Breast cancer is a heterogeneous disease and understanding molecular dysregulations has resulted in identification of novel therapeutic targets. The development of kinase inhibitors and Her2 targeting monoclonal antibodies led to increased survival rates among breast cancer patients, in particular in patients with local disease (2). However, relapse and metastases remain a hurdle to therapy and are the most common causes of death among women with breast cancer (3). Metastases derive from disseminated tumor cells, where epithelial mesenchymal transition (EMT) is a required process for the occurrence of metastasis at distant sites (4). Which cells in particular undergo this process and have greater potential to metastasize is not fully understood. Cancer stem cells (CSC) have been proposed to be one source of metastasis in breast cancer, and circulating tumor cells in patients with metastatic breast cancer express EMT markers and display a stem cells phenotype (5,6).

In recent years, immunotherapy has become of interest in cancer therapy and has been successfully used to treat metastatic disease (7). The term immunotherapy summarizes diverse modalities of immune-based treatments, including checkpoint blockade, vaccines and adoptive transfer of immune cells. Checkpoint blocking antibodies targeting PD-1 and CTLA-4 are currently in

clinical trials (NCT02129556, NCT02892734) for metastatic breast cancer. CTLA-4 and PD-1 blockade exhibits two distinct mechanisms of action with PD-1 blockade restoring function of anergic T cells and CTLA-4 expanding the T cells repertoire (8).

Until now, therapeutic vaccines in cancer have been less successful. The success of antitumor vaccines is highly dependent on the choice of antigen and co-stimulating agents as well as mode of delivery (9). Vaccines have the great potential to boost pre-existing anti-tumor immunity, and to activate tumor eliminating effector cells. For breast cancer, several different vaccines targeting Her2 are currently in clinical trials (NCT01570036, NCT01152398, NCT02276300, NCT00194714), and we have conducted a pilot trial with a full length non-transforming Her2 DNA (10). For treatment of metastatic breast cancer, it is of particular interest to target antigens expressed on CSC and metastasizing cells.

Cripto-1 (Cr-1) is an onco-fetal protein re-expressed in the majority of human tumors, including breast cancer (11). In breast cancer, Cr-1 expression in tumor cells is negatively correlated with survival (12). Cr-1 is a GPI-anchored cell surface protein essential in embryonic development. The protein co-localizes with several receptors and is involved in Nodal, TGFβ, and Wnt/βcatenin signaling among others (13). In tumors, Cr-1 has been shown to be involved in cell proliferation and migration, EMT and angiogenesis (14). In addition, Cr-1 plays an important role in the maintenance of embryonic stem cells and is a target gene of the transcription factors Nanog and Oct4 in stem cells. Indeed, Cr-1-positive cells were found to be Nanog- and Oct4-positive and able to form spheres in vitro (15). Studies on CSC in melanoma and prostate cancer have

shown that Cr-1 expression is associated with an undifferentiated phenotype (15,16). The expression of Cr-1 on CSC together with its role in intracellular EMT signaling makes it a potential antigen for metastasis and CSC targeting in breast cancer.

We have previously shown that vaccination against Cr-1 elicits a protective immune response in C57BL/6 mice and results in reduced tumor burden upon subcutaneous challenge with murine melanomaB16F10 cells. Intravenous (i.v.) challenge with B16F10 in mice vaccinated with plasmids encoding murine Cr-1 (pmCR) resulted in significant reduction of lung metastatic foci (17).

Here we describe that vaccination induced an anti-Cr-1 directed humoral response that protects from metastasis burden in the aggressive orthotopic 4T1 and the spontaneous BALB-neuT breast cancer mouse models. Further, we show Cr-1 specific clearance of breast CSC *in vivo*. Anti-Cr-1 vaccination could potentially be of great benefit for patients with breast cancer, reducing the risk of relapse and disease progression.

Material and methods

Cell lines

4T1 luciferase expressing cells (4T1) TS/A and D2F2 cell lines was maintained in RPMI 1640 supplemented with L-glutamine and 10% heat-inactivated FBS (Life technologies). TUBO cell line (18) was maintained in DMEM supplemented with 20% FBS (Sigma-Aldrich). Murine Cripto-1(mCr-1)-expressing 4T1 (4T1mCr-1) cells were generated by transducing 4T1 cells with lentiviral particles (Amsbio). mCr-1-expressing cells were FACS sorted, see Flow cytometric analysis, and further selected with Geneticin (Life technologies).

Spheroid culture

TUBO and 4T1 single-cell suspensions were seeded in DMEM-F12 supplemented with 20 ng/ml EGF, 20 ng/ml FGF, 5 µg/ml insulin, 0.4 % BSA (Peprotech, Sigma Aldrich) at a concentration of 6 x 10^4 cells/ml in ultra-low attachment plates (Corning). The resulting spheroids were monitored daily and passed using enzymatic and mechanical dissociation every 3-5 days. Cells were re-seeded at 6 x 10^4. Spheroid cultures were passaged 3 times and passage 1 (P1), 2 (P2) and 3 (P3) were collected for further experiments.

Mice

BALB/c mice were either purchased from ScanBur and maintained at the Department of Microbiology, Tumor and Cell Biology (Karolinska Institutet, Stockholm, Sweden) or bred and maintained at the Molecular Biotechnology Center (University of Torino, Torino, Italy). BALB-neuT mice were bred and maintained at the Molecular Biotechnology Center (University of Torino, Torino, Italy). Mice were handled in accordance to regional Animal ethics committees (Stockholms Norra Djurförsoksetiska Nämnd Avdelning 2, Sweden N426/11,

N239/14; University of Torino ethical committee authorization number 837/2015-PR).

Plasmid

Mouse Cr-1(NM_011562.2) encoding plasmid was generously donated by Bianco C et al., (NCI NIH Bethesda) (19) and the coding sequence was subsequently cloned into the pVAX11 vector (Invitrogen) to obtain pmCr-1. pmCr-1 and pVAX11 were expanded in *E.coli* (TOP10, Invitrogen) grown in LB medium containing Kanamycin selection (50 μg/ml). Plasmids were purified using GigaPrep Endofree Kit (Qiagen).

4T1mCr-1 orthotopic model

BALB/c mice were vaccinated at 8 and 10 weeks of age by intradermal injection of 40 μg of plasmid in PBS followed by electroporation with plate electrodes (IGEA). Electroporation protocol has been previously described (17). In week 12, $2x10^5$ 4T1mCr-1 cells diluted in 50 μl PBS were injected into the mammary fat pad. Tumors were measured by palpation twice per week and tumor volume was calculated using the formula (π/6) x L x W x H (20). Mice were sacrificed 3 weeks after tumor challenge and primary tumors were excised and weighed. Tumors were snap frozen in OCT. For lung colony formation assay, single-cell suspensions were prepared from harvested lungs, seeded in 15 cm dishes and cultured in RPMI supplemented with L-glutamine, 10% FBS, 1% PenStrep, 6-Thioguanine (Sigma Aldrich). Medium was changed every 3-4 days. Upon colony formation, cells were fixed with 4% formaldehyde and stained with hematoxylin. Colonies were evaluated by counting.

BALB-neuT model

BALB-neuT mice were vaccinated with prime and boost at 10 and 12 weeks of age, respectively, by intramuscular injection of 50 μg of plasmid in saline. The injection was followed by electroporation using IGEA array needle electrode. Mice were inspected weekly for the presence of tumors, whose dimension was reported as mean tumor diameter. When mice reached a total number of 10 mammary tumors, or a tumor reached a threshold size of 10 mm mean tumor diameter, mice were progressively culled, lungs were harvested and fixed in paraffin followed by staining with hematoxylin and eosin. Lung metastases were counted on a Nikon SMZ1000 stereomicroscope (Mager Scientific). The metastatic index was calculated by dividing the number of metastatic foci by the sum of the diameter of all primary lesions.

TUBO P3 model

BALB/c mice were vaccinated at 8 and 10 weeks of age by intramuscular injection of 50 μg plasmid in saline. The injection was followed by electroporation using IGEA array needle electrode. Two weeks after the second vaccination mice were challenged subcutaneously (s.c.) with $2x10^4$ TUBO P3 spheroids as described (21). Mice were inspected weekly for the presence of the tumor, whose dimension was reported as mean tumor diameter. Overall survival was reported as the time required by the tumor to reach the threshold of 10 mm mean tumor diameter, according to ethical guidelines.

Antibodies

See supplemental table 1.

Serum

Serum was collected for analysis and *in vitro* studies 2 weeks after the second vaccination. Sera from all mice in each group within one experiment were pooled.

Western Blot

Cell lysates were prepared from fresh cell culture or snap frozen cell pellets stored at -80°C with 1 M RIPA buffer (150mM NaCl, 1% Triton-X 100, 0.5 % sodium deoxycholate, 0.1% SDS, 50 mM NaF, 50mM Tris-HCl ph 7.4) and 1x protease (Roche). Protein concentrations were determined with Pierce BCA protein assay (Thermo Scientific) prior to loading onto gel. 20 µg protein lysates were reduced with 1x NUPAGE Reducing agent (Invitrogen) and 1x NuPage LDS Sample Buffer (Invitrogen) and loaded on 10% NuPAGE Bis-Tris acrylamide gels (Invitrogen). Proteins were transferred to PVDF membrane with methanol wet-transfer. Primary antibodies were incubated overnight at 4°C and secondary antibodies for 1h at RT. Membranes were developed using Pierce ECL Western Blotting Substrate reagent kit. Luminescence was detected using LAS-1000 CCD camera system (Fujifilm, Tokyo, Japan).

Flow cytometric analysis

For flow cytometric analysis, single cell suspensions were prepared and $2x10^5$ cells were stained per sample. Cr-1 specific antibodies in serum of pmCr-1 vaccinated mice were detected by cell surface staining of 4T1mCr-1 with serum from pmCR-1 vaccinated mice. For FACS sorting, transduced 4T1 cells were first stained with pmCr-1 serum and then with anti-mIgG-PE. pVAX1 serum was used as a negative control staining. For IgG subclass analysis, 4T1mCr-1 binding serum derived antibodies were detected with anti-mIgG-FITC, anti-mIgG1-FITC, anti-IgG2a-FITC and anti-IgG2b-FITC. For unstained control, cells were only

stained with secondary antibodies. Percentage of IgG1, IgG2a and IgG2b were calculated by dividing mean fluorescent intensities (MFIs) by the sum of MFI for IgG1, IgG2a and IgG2b after subtraction of MFI of unstained cells. All samples were acquired either on LSRII (BD) or Novocyte (ACEA) and analyzed using FlowJo (Tree Star).

In vivo imaging

In vivo imaging was done with IVIS SpectrumCT (PerkinElmer) using D-Luciferin (Life Technologies). 5 µg D-Luciferin per gram mouse was injected i.p. and allowed to disseminate in the mouse for two minutes followed by anesthesia with Isoflurane at 3% for three minutes prior to transfer onto the heated, 37°C, SpectrumCT platform (Perkin Elmer) for imaging and analyzed using Living Image Software (Perkin Elmer).

Lung colony assay

Lungs from 4T1mCr-1 bearing mice were harvested and kept in cooled PBS supplemented with 10% FBS. Lungs were individually mechanically and enzymatically digested in RPMI supplemented with 5% FBS, 2 mg/ml Dispase, 100 µg/ml DNase I, 200 µg/ml Collagenase IV for 30 min at 37°C. Cell suspension was filtered using a 70 µm filter (Fisher Scientific). Removal of red blood cells was done using RBC lysis buffer (BioLegend) and followed by suspension in supplemented RPMI-1640 media containing 6-Thioguanine (60 µM) and seeded in 150 mm cell culture dishes (Corning). After 10 days, cells were washed with PBS, followed by formaldehyde fixation and Hematoxylin Harris (VWR, 351945S) staining for 5 minutes. Primary tumors were excised and weighed. To evaluate lung metastasis, colonies were enumerated and metastatic index was calculated, MI = number of colonies/primary tumor weight.

Antibody dependent cellular cytotoxicity (ADCC) assay

4T1mCr-1 and 4T1 cells were harvested and labeled with ^{51}Cr (Perkin Elmer). After labeling, target cells were incubated for 10 minutes at 4°C with 10 µl of serum from pmCr-1 or pVAX1 vaccinated mice. $5x10^3$ cells per well were then plated in 96-well plates without washing. wt BALB/c mice were sacrificed and splenocytes isolated. NK cells were purified with magnetic beads by DX5-positive selection (Miltenyi Biotech). NK cell fraction and negative fraction were titrated onto target cells. 25 µl of co-culture supernatant were harvested after 4 and 16 h onto LUMA plates (Perkin Elmer). Radioactivity was detected in beta-counter (Perkin Elmer).

Statistical analysis

Data was analyzed with Prism 7 (GraphPad software). All in vivo data is shown as mean ± SD. Tumor growth, metastatic index and tumor growth rate were compared using Mann-Whitney test. Tumor weights were compared with unpaired t-test. Survival data were compared with log rank test. For NK cell cytotoxicity, 5 independent experiments are displayed and compared with paired t-test. p-values < 0.05 were considered statistically significant.

Results

<u>Vaccination with mouse Cripto-1-encoding DNA plasmid reduces metastatic burden and primary tumor growth in 4T1 metastasis model</u>

We aimed to understand if vaccination with pmCr-1 would elicit a protective immune response in a model of murine metastatic breast cancer. We screened four mouse mammary carcinoma cell lines on BALB/c background for Cr-1

298 expression by western blot. Weak bands of Cr-1 were found in 4T1, TUBO and
299 TS/A, while D2F2 was negative for mCr-1 expression (Supplemental Fig. 1). As a
300 first approach to establish the protective potential of mCr-1 vaccination-induced
301 immune responses over the dissemination of mammary cancer cells in BALB/c
302 models, we generated a stable mCr-1 expressing 4T1 transfectant (4T1mCr-1),
303 which was used as a model for spontaneous lung metastasis (Supplemental Fig.
304 1). BALB/c mice were vaccinated with pmCr-1 or control pVAX1 plasmids prior
305 to implantation of 4T1mCr-1 cells into the mammary fat pad. Primary tumor
306 growth was evaluated by *in vivo* luciferase activity detection at day 14 (Fig. 1A)
307 and twice per week through palpation (Fig. 1B). At day 23 after tumor
308 inoculation, mice were sacrificed and primary tumor weight measured (Fig. 1C).
309 Primary tumor size and weight were significantly reduced in pmCR-1- compared
310 to pVAX1-vaccinated mice. Furthermore, pmCR-1 vaccination greatly reduced
311 spontaneous metastasis to the lungs as evaluated by a colony formation assay
312 (Fig. 1D). Cr-1 vaccination results in anti-tumor immunity capable of controlling
313 tumor growth and inhibiting metastatic spread.

314 <u>Cripto-1 specific humoral response</u>

315 It was previously shown that DNA vaccination in BALB/c mice can elicit a
316 humoral response (22). We therefore evaluated the humoral response after
317 vaccination with pmCR-1(23,24). Serum of pmCR-1-vaccinated mice was found
318 to contain antibodies that stained specifically mCr-1 expressing 4T1 cells (Fig.
319 2A), while no signal was observed on 4T1 cells. We found that the majority of
320 these antibodies belonged to IgG2a and IgG2b subclasses (Fig. 2B). In mice, these

subclasses are responsible for mediating ADCC by NK cells, macrophages and neutrophils.

Cripto-1 directed antibody dependent cellular cytotoxicity

NK cells play a major role in the success of antibody-based immunotherapy. For several clinically successful therapeutic antibodies, including anti-Her2, anti-EGFR and Anti-CD20, NK cells mediated cytotoxicity is a known mechanism of action (25).

To confirm that Cr-1 specific antibodies can mediate ADCC, we tested if serum from pmCR-1-vaccinated mice increases cytotoxicity by NK cells. NK cells were purified from BALB/c splenocytes with magnetic bead selection and co-cultured with 4T1mCr-1 or 4T1 cells in the presence of pmCr-1 or pVAX1 serum. We found that pmCr-1 serum significantly increased lysis of 4T1mCR-1 cells by NK cells (Fig. 2C, D). No cytotoxic activity was detected by splenocytes depleted of NK cells (data not shown). To show that ADCC is Cr-1 specific we co-cultured NK cells with 4T1 cells in presence of serum from pmCr-1 and pVAX1 vaccinated mice. No difference in 4T1 lysis by NK cells was observed in presence of pmCr-1 serum compared to pVAX1 serum (Fig. 2E).

Reduced lung metastasis after vaccination in the BALB-neuT mouse model

We additionally wanted to test if pmCr-1 vaccination has therapeutic effect in a more clinically relevant model (26). The BALB-neuT mouse model is genetically engineered to develop spontaneous cancerous lesions in the mammary tissue. We evaluated Cr-1 expression in the breast tumors of the model and only found low expression in tumors of 8 mm mean diameter (Suppl. Fig. 2) with no Cr-1

346 expression in smaller tumors. Mice were vaccinated at 10 and 12 weeks of age,
347 but this did not result in difference in tumor outgrowth (data not shown) nor did
348 it affect tumor incidence in this mouse model (Fig. 3A). Consequently, we did not
349 observe survival benefits (Fig. 3B) until mice were sacrificed according to the
350 ethical regulations. At sacrifice, lungs were evaluated for the presence of
351 metastasis. Micrometastases derived from the primary tumors can be found in
352 the lungs within 8 weeks of primary tumor occurrence (27).
353 Lungs from pmCR-1- and pVAX1-vaccinated mice were sectioned, stained with
354 hematoxylin and eosin and metastatic foci enumerated. We found that
355 metastatic burden was significantly reduced in pmCR-vaccinated BALB-neuT
356 mice (Fig. 3C). We observed that both the number of foci as well as metastatic
357 size was reduced (Fig. 3C, D).
358
359 Vaccination results in protective immune response targeting cancer stem cells
360 Since targeting Cr-1 inhibits metastases, which can be caused by CSC, and Cr-1
361 expression has previously been associated with CSC in melanoma, colon and
362 breast cancer CSC (5,28-30). We therefore wanted to evaluate if Cr-1 vaccination
363 elicits a protective immune response against Cr-1 expressing CSC. It has been
364 shown that the murine mammary carcinoma cell line TUBO acquires CSC
365 phenotypic markers when passaged 3 times as spheres (P3 TUBO cells) (21,31).
366 Over the three passages in spheroid culture of TUBO, we observed a gradual
367 increase in expression of Cr-1 (Fig. 4A). These TUBO P3 cells were s.c. injected in
368 vaccinated BALB/c mice. We observed a decreased growth rate as a result of
369 pmCR-1 vaccination. The time to reach the mean tumor size of pmCr-1 group (4
370 mm in diameter) was significantly longer in pmCr-1- compared to pVAX1-

vaccinated mice (Fig. 4B). In addition, we found that 3 out of 11 mice in the pmCr-1 group were completely tumor free more than 60 days after tumor inoculation (Fig. 4C). In comparison, all mice in pVAX1 treatment group developed tumors within 47 days. Vaccination targeting Cr-1 also resulted in a trend towards improved survival (p=0.078) (Fig. 4D).

Discussion

The metastatic process of tumors is complex and until today not fully understood. Two critical cellular processes are crucial for the occurrence of metastasis, which are EMT and mesenchymal-epithelial transition (MET) (32). EMT enables cells to survive without cell-cell contact, to migrate and to extravasate from the primary tumor. At the site of distant metastasis MET is required for cells to establish metastatic colonies and grow out. Cr-1 is expressed in cells undergoing EMT and higher expression of this protein has been found in more aggressive types of human breast cancer (12,33).

We have previously reported that Cr-1 is an immunogenic antigen and that vaccination against Cr-1 results in protective anti-tumor immune responses against murine melanoma. In this model, a strong protective effect against pulmonary metastases was observed upon i.v. challenge with metastatic B16F10 cells (17). It is of considerable importance to study the vaccine in a model recapitulating the complete metastatic cascade from tumor cells undergoing EMT at the primary tumor site to MET at the site of metastasis. We therefore chose to study this process in the 4T1 orthotopic breast cancer model and in Her2 transgenic BALB-neuT mice. When 4T1 cells are orthotopically injected into the mammary fat pat, they spontaneously metastasize (34,35). Similarly, the

BALB-neuT mice develop autochthonous mammary tumors that early metastasize and colonize the lungs (27). These models enable the study of EMT and MET *in vivo*. Due to low endogenous Cr-1 expression, we overexpressed murine Cr-1 in 4T1 cells (Suppl. Fig. 1). We observed that Cr-1 vaccination reduced metastatic burden in both the orthotopic 4T1 and the spontaneous BALB-neuT breast cancer model (Fig.1D and 3C, D). Control of the primary tumor was only seen in the Cr-1 overexpressing 4T1 model. This is in line with the lack of Cr-1 expression in the primary tumors of the BALB-neuT model (Suppl. Fig. 2).

We observed that the pmCr-1 vaccination induced an anti-mCr-1 humoral response in the BALB/c mouse model, while we were not able to identify anti-mCr-1 antibodies in the pVAX1 vaccinated mice (Fig. 2A). Further did the majority of Cr-1 targeting antibodies belong to the IgG2a subclass (Fig. 2B), able to bind murine activating Fcγ receptors with relatively high affinity. In view of these results, we aimed at understanding the role of NK cells in Cr-1 vaccination-induced tumor control. Collectively, our data pointed at a critical role for NK mediated ADCC in pmCr-1-vaccinated mice (Fig. 2). These results are reminiscent of our earlier findings, where we have shown that Her2-vaccination in BALB/c mice initiated a humoral anti-Her2 immunity and consequently killing of Her2-positive tumor cells by NK cells (22). *In vitro* cytotoxicity data demonstrated that lysis of Cr-1 expressing cells by NK cells was increased in the presence of serum from pmCr-1-vaccinated mice (Fig. 2C and D), pointing at a major role for ADCC in the tumor elimination. Hereby we were able to show one mechanism of vaccination-induced tumor elimination by NK cells.

In a previous study we have shown that anti-Cr-1 vaccination in C57Bl/6 mice induced an *in vitro* detectable cytotoxic T cell response (17). After vaccination, Cr-1 specific cytotoxic T cells have not been detected *in vitro* in BALB/c splenocytes (data not shown). Although these results do not entirely rule out a possible role for T cells in the observed *in vivo* tumor protection, they argue for a difference in immune response between BALB/c and C57Bl/6 mice upon DNA vaccination. In a study performed by Radkevich-Brown et al. Her2 DNA vaccination elicited a humoral immune response in Her2 transgenic BALB/c mice. In a direct comparison, Her2 vaccination induced significantly lower levels of Her2-specific antibodies in C57Bl/6 mice than in the BALB/c mice (36). The differences observed are to be explained with the genetic differences of the mice strains and can be translated to our findings in the BALB/c and C57Bl/6 mice after Cr-1 DNA vaccination (17).

Cr-1 expression is potentially limited to CSC, a few cells undergoing EMT in the primary tumor, and metastasizing cells. De Castro et al. recently described Cr-1 expression in EMT-like areas in the JygMC(A) breast cancer model. In contrast, no Cr-1 expression was detected in metastatic lesions in the lung (37). Vaccination against Cr-1 could potentially interrupt the metastatic process at an early stage and thereby prevent the establishment of metastases at distant sites.

In CSC of several tumor types, Cr-1 expression has been confirmed (15,38,39). We have found that spheroid cultures of murine breast cancer cells, which are considered to be enriched in CSC, upregulate Cr-1 expression (Fig 4A) (21,30,31). Subcutaneously injected TUBO P3 cells grew out in all BALB/c mice within 6 weeks after injection. After vaccination against Cr-1, 27% of mice did not develop tumors (Fig. 4C). In the remaining mice, we observed a reduced

tumor growth rate (Fig. 4B and C). Immune responses induced by Cr-1 vaccination specifically target Cr-1-positive CSC and control tumor burden.

In patients, high levels of Cripto-1 expression in the tumor have been associated with decreased survival and could be correlated to advanced disease (12). In addition, Cripto-1 has been found in the serum of breast cancer patients, suggesting its potential function as a biomarker (40). For lung cancer, it was reported that serum levels of Cripto-1 correlated with tumor stage (41). These reported clinical findings associate increase expression of Cripto-1 with metastasis and worse survival in breast cancer patients.

It is crucial for patient survival to eliminate tumor cells that can cause relapse and metastasis to potentially prolong patient survival. New therapeutic strategies, which can specifically target both CSC and metastases, have the ability to reduce the risk of relapse and disease related death in cancer patients. Immune targeting therapies have shown a great potential in treatment of metastatic diseases (42). It is crucial to identify novel immunogenic antigens that can be targeted by immunotherapies. We propose that Cripto-1 is a suitable candidate for immunotherapy in breast cancer patients, targeting a different subset of breast cancer cells than in our previous Her2 DNA vaccine clinical trial (10). We have shown that targeting Cripto-1 in breast cancer mouse models reduced metastasis and targeted CSC. For patients, a DNA vaccine targeting Cripto1 could potentially translate into increased disease free and overall survival.

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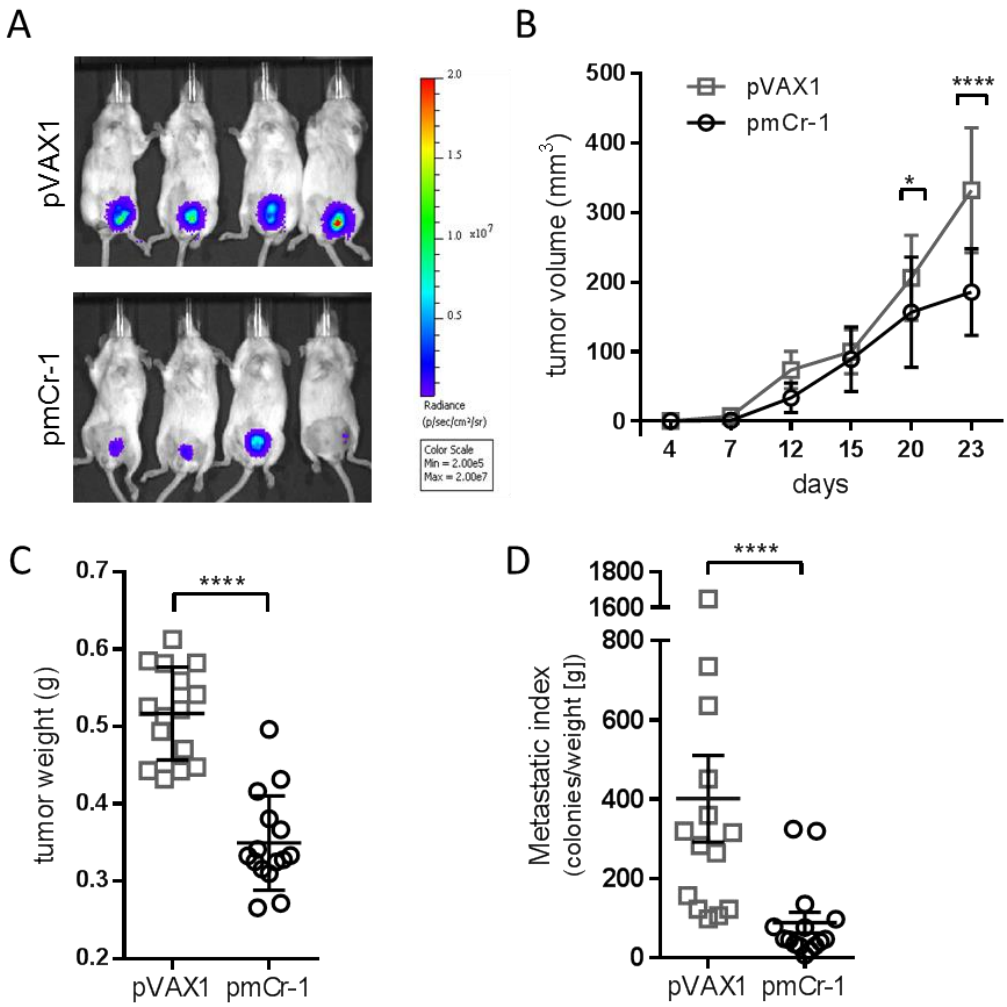
Figure 1

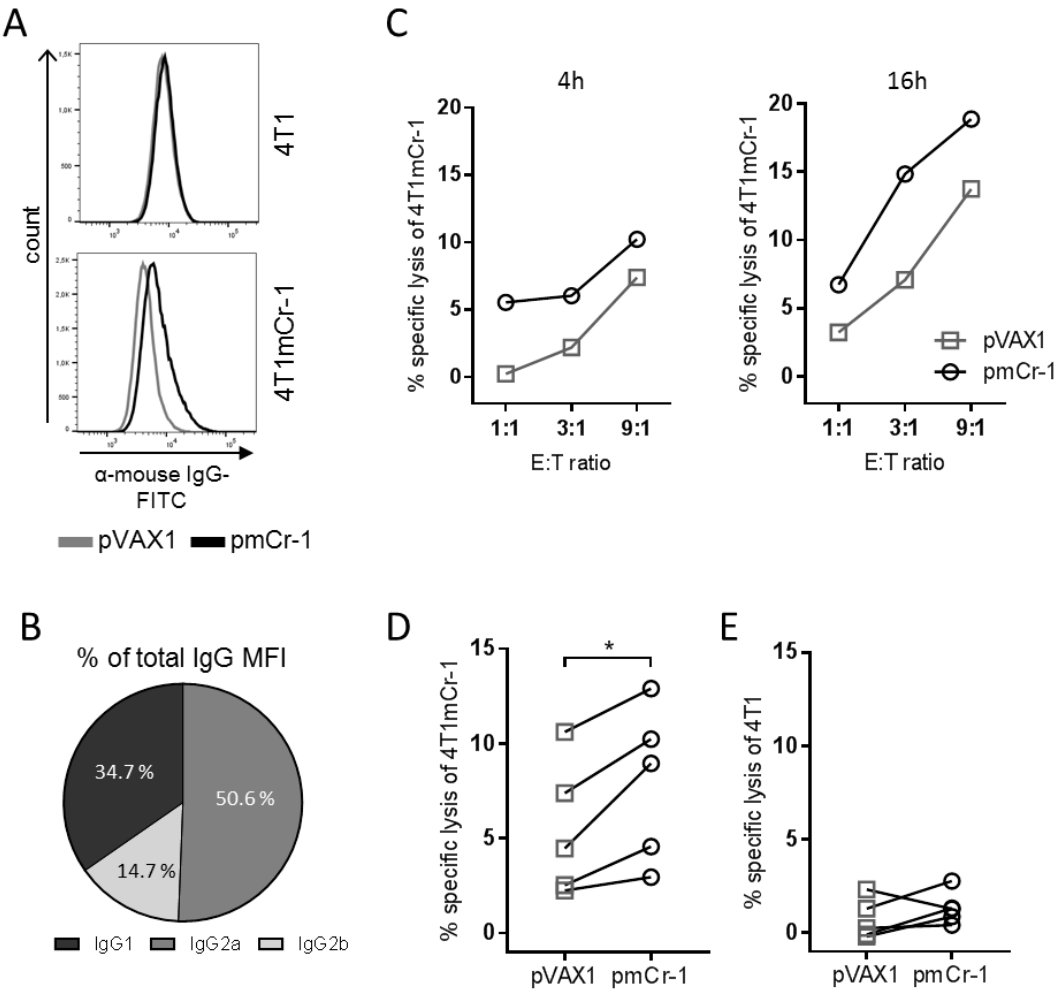


Tumor growth and metastatic spread in orthotopic 4T1mCr-1 breast cancer model

Orthotopic injection of 2x105 4T1mCr-1 cells in pmCr-1- or pVAX1- vaccinated BALB/c mice. Mice were sacrificed on day 23 after tumor inoculation. **A**, Luciferase expression at day 14 after tumor inoculation. 4 representative mice are displayed. **B**, Volume of primary tumors. Mice in pVAX1 (n=5) and pmCr-1 (n=5) group were palped twice per week until experimental endpoint on day 23. Error bars represent standard deviation; * p=0.0321, **** p<0.0001 (Mann-

Whitney test). **C**, Primary tumor weight at day 23. Error bars represent standard deviation; **** $p<0.0001$ (unpaired t-test). **D**, Single cell suspension of lung tissue was seeded in petri dish and cultured in selection medium. At day 10, colonies were fixed and counted. Metastatic index (MI) was calculated by MI= number of colonies/primary tumor weight. Error bars represent standard deviation; **** $p<0.0001$ (Mann-Whitney test).

Figure 2

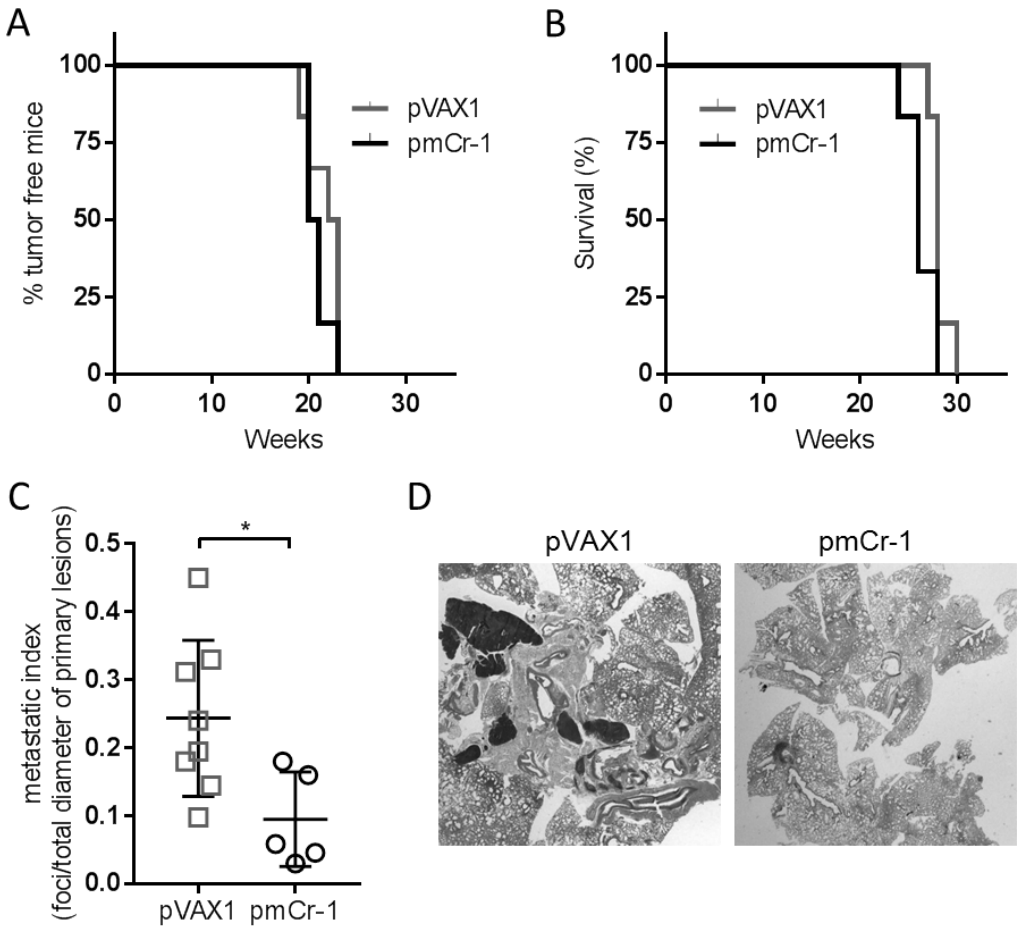


Humoral response induced by pmCr-1 vaccination in BALB/c mice

BALB/c mice were vaccinated with pmCr-1 or pVAX1. Two weeks after the boost vaccination, serum was collected for analysis. **A**, 4T1mCr-1 and 4T1 cells were incubated with serum from pmCr-1 and pVAX1. Surface binding serum antibodies were detected with anti-mIgG-FITC antibody. Cells were analyzed on flow cytometer. **B**, Subclasses of antibodies in pmCr-1 serum binding Cr-1 were detected with secondary anti-mIgG1-FITC, anti-mIgG2a-FITC, anti-mIgG2b-FITC. Cells were analyzed by flow cytometry. **C**, NK cells cytotoxicity against 4T1mCr-

650 1 cells in the presence of pmCr-1 or pVAX serum. Assay supernatants were
651 harvested after 4h and 16h for analysis. **D**, NK cell cytotoxicity against 4T1mCr-1.
652 Summary of 5 individual experiments after 4h co-culture at 9:1 effector to target
653 ratio; * p=0.0158 (Paired t test). E, NK cell cytotoxicity against 4T1. Summary of
654 5 individual experiments after 4h co-culture at 9:1 effector to target ratio.
655

Figure 3

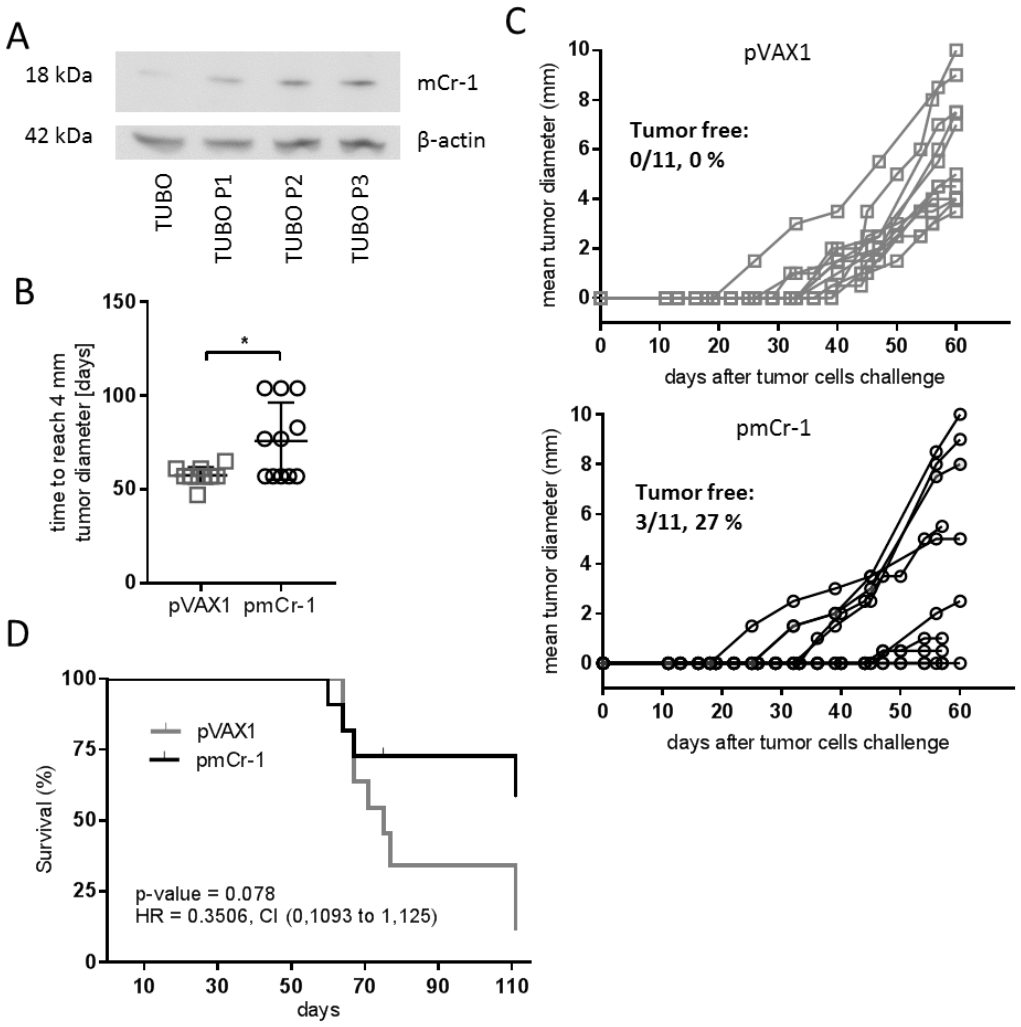


Metastatic spread in Her2/neu driven spontaneous breast cancer model BALB-neuT

BALB-neuT mice were vaccinated at 10 weeks and 12 weeks with pmCr-1 or pVAX1. Mice were followed over time and sacrificed upon ethical endpoint. **A**, Tumor incidence in pVAX1 (n=6) and pmCr-1 (n=6) vaccinated BALB-neuT mice. **B**, Survival of pVAX1 (n=6) or pmCr-1 (n=6) vaccinated BALB-neuT mice. Mice were sacrificed upon ethical endpoints. **C**, Metastatic burden in the in pVAX1 (n=5) and pmCr-1 (n=5) mice. Metastatic index is calculated by MI=number of foci/sum of the diameter of all primary lesions. Error bars represent standard deviation; * p=0.021 (Mann-Whitney test). **D**, Light

668 microscopy image of the lung sections after hematoxylin and eosin staining, 10x
669 magnification.
670

Figure 4



Vaccination induced immune response is targeting breast cancer stem cells

P3 TUBO cells were s.c. injected in pmCr-1 or pVAX1 immunized BALB/c mice. **A**, Western Blot for Cr-1 in spheroid passaged TUBO cell line. **B**, Tumor growth rate in pmCr-1 (n=11) and pVAX (n=11) vaccinated mice. Error bars represent standard deviation; * p=0.0453 (Mann-Whitney test). **C**, Individual tumor growth curves for pmCr-1 and pVAX until day 61. **D**, Survival curves for mice immunized with pmCr-1 or pVAX1 after s.c. challenge with TUBO P3. p=0.078 (Mantel-Cox test).